Chemokines in neuroimmunology

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>Summary and discussion<

Chemokines and neuron-glia communication

The view of the CNS being an immune-privileged site has changed dramatically in the last decades. It is nowadays acknowledged that brain resident cells, particularly astrocytes and microglia, are immunocompetent cells [2,23]. Activation of these glial cells in response to neuronal challenges initiates and contributes to the development of neuroinflammation under various neurodegenerative conditions. Thus, pathways that mediate communication between neurons and glial cells are likely to exist [1].

The last years, reports on the localization and function of chemokines in the CNS have been accumulating. As a result, chemokines are now considered to play a key-role in different CNS processes occurring under normal healthy conditions, but also in pathology. It has been recognized that, besides their classical function of recruiting leukocytes chemotaxis across the BBB, thereby mediating neuroinflammation, chemokines are also involved in neurodevelopment, neuroprotection and synaptic transmission. Moreover, the fact that neurons and glial cells express chemokines as well as chemokine receptors suggests, that chemokines are mediators of neuron-glia communication, which may play a crucial role in the onset and outcome of neuropathology [5,6,8,9,15,29,38,45].

CCL21 and its receptors in the CNS

CCL21 is a chemokine that is expressed at high levels in the peripheral secondary lymphoid tissues. Therefore, this chemokine plays an important role in the homing of dendritic cells (DCs) and T-cells to the lymphnodes, and the coördination of immune responses [22,27,58]. The first study addressing the presence of CCL21 in the brain described rapid induction (within 6 hours) of neuronal CCL21 mRNA in an *in vivo* ischemia mouse model [14]. This fast expression suggests that CCL21 acts as an early signal coming from endangered neurons in order to activate glial cells. In particular microglia might be targets for CCL21 since these are the first cells that are rapidly activated at early stages of neuropathology. The aim of this thesis was to provide evidence for a neuron-glia communication pathway mediated by CCL21 and its receptors.

Endangered neurons express and release CCL21

In the already mentioned study by Biber et al., detection of CCL21 during ischemia was limited to the level of mRNA transcripts. Therefore, the presence of CCL21 protein needed to be verified. The **second** chapter of this thesis described expression of CCL21 protein in primary neuronal cell cultures and organotypic hippocampal slice cultures that were subjected to treatment with toxic concentations glutamate. Exposure of these cultures to glutamate resembles, to a certain degree, the excitotoxic environment resulting from ischemia *in vivo* and thus provided comparable

and reproducible *in vitro* and *in situ* models.

Detection of CCL21 protein shortly after glutamate treatment thus corroborated the findings on CCL21 mRNA expression. Moreover, CCL21 protein was found to be present in (pre) apoptotic neurons, determined by evaluating nuclear morphology and caspase-3 immunocytochemistry. During analysis of glutamate-treated neuronal cultures and organotypic slices, CCL21 immunoreactivity was observed in Golgi vesicle-like structures and in the axons. These observations were verified by electron microscopy, which clearly showed CCL21 immunoreactivity in vesicles at the axon hillock, throughout the axons, in pre-synaptic structures and in the synaptic cleft. In addition, microglia and HEK293 cells transfected with CCL21 receptors showed chemotaxis in response to supernatants obtained from glutamate-stimulated neuronal cultures. Thus, these data indicate that CCL21 is released from endangered neurons at the synapse and is subsequently able to elicit functional responses in specific target cells.

Glial cells express CXCR3 and exhibit functional responses to CXCL9, CXCL10 and CCL21

To provide further evidence for CCL21-mediated neuron-glia signaling, the presence of receptors for this chemokine was examined in glial cells. The main receptor for CCL21 is CCR7, belonging to the CC chemokine receptor group [19,58]. However, CCR7 was never detected in any CNS cell type [14]. Another receptor belonging to the CXC receptor subgroup, namely CXCR3, has also been shown to bind CCL21 and exhibit functional activity [53]. Interestingly, this receptor also binds CXCL9 and CXCL10, chemokines that are consistently found upregulated in different neurodegenerative conditions, like ischemia, EAE and MS [10,25,44,50-52,56,57].

In the **third** chapter, expression of CXCR3 was examined at both the RNA and protein level in astrocytes and microglia from mice as well as humans. Moreover, functional activity of CXCR3 in these cells was shown by means of chemotaxis assays and calcium imaging in response to its main ligands CXCL9 and CXCL10. It was previously reported that CCL21 activates murine microglia as evidenced by the induction of migration, calcium transients and altered electrophysiology. In addition, cross-desensitization experiments and studies in CXCR3 and CCR7 knockout mice, showed that these CCL21-induced effects are most likely mediated by CXCR3 [14,46].

Nevertheless, binding of hCCL21 to hCXCR3 was never observed in recombinant expression systems, suggesting that interaction between CCL21 and CXCR3 is a phenomenon existing only in mice [32,57]. Because studies addressing this issue were all performed in hCXCR3 transfected cell lines and not in (CCR7 lacking) primary cells, we examined whether human microglia showed functional activity in response

to CCL21.

In the **fourth** chapter it was demonstrated that cultured primary human microglia cells, expressing CXCR3 and lacking CCR7, respond to CCL21 with migration. Similar to murine microglia, this effect was most likely mediated by CXCR3, as evidenced by lack of responses to another CCR7 ligand, CCL19, and cross-desensitization by CXCL10.

Interactions between CCL21 and CXCR3 represent a possible neuron-microglia signaling pathway

Taking together, on the one hand the chemokine CCL21 was found to be expressed and released by damaged neurons. On the other hand, a CCL21 receptor, CXCR3, was detected in both astrocytes and microglia. Moreover, both cell types exhibited CXCR3-mediated functional activity. Since these findings provide at least the basic requirements for neuron-glia communication (a signal and the receptor), they are in support for the hypothesis that CCL21 is involved in neuron- (micro) glia communication.

Furthermore, transport of CCL21 along axonal processes offers an explanation for the fact that, in certain lesion models, microglia activation can be observed at great distance from the actual site of neuronal damage [11,13,18,35]. Thus, endangered neurons express CCL21, anterogradely transport this chemokine along the axon and release it at synapses where microglia are subsequently attracted and activated.

So far, microglia are the only CNS cells that were demonstrated to respond to CCL21 via CXCR3. Although other cell types, exhibiting similar CCL21-CXCR3 mediated activation, cannot be excluded yet, it is tempting to speculate that CCL21 released from endangered neurons would only be detected by microglia and no other CXCR3-expressing cells. In line with this speculation are the recent findings that showed no differences in the activation of astrocytes in response to brain damage in CXCR3 deficient mice, whereas microglia activation was significantly impaired (Rappert et al. manuscript submitted).

It has been recognized that microglia activity is controlled by neurons. Accordingly, lack of signals normally provided by healthy neurons, would trigger microglia activation. Examples of such signals are neuronal firing, neurotrophins, CX3CL1, CD200 and CD22, which were demonstrated to be provided by healthy neurons and suppress microglia activation [30,39,59]. This thesis provides evidence for a novel inducible neuronal signal, which directly activates microglia and may therefore play a crucial role in initiating neuroinflammation in response to neuronal damage.

As already mentioned, CXCL10 has also been found expressed in neurons during neurodegeneration, therefore this chemokine may as well act as a communication

signal activating CXCR3 expressing glial cells. Nevertheless, CXCL10 has, so far, only been detected in neuronal somata and not in axonal processes (own unpublished findings)[56], which indicates CXCL10 to be a signal attracting glial cells in the near surrounding of the damaged neuron.

In addition, CXCL10 is also expressed in both astrocytes and microglia [10,25,44,50-52], thus suggesting possible glia-glia communication pathway via CXCL10-CXCR3 interactions. This might be an important event in sustaining and spreading the inflammatory response by attracting local glial cells and leukocytes across the BBB.

Noteworthy is the fact that expression of CXCR3 by neurons has also been reported and interestingly, CXCL10 appears to modulate neuronal activity mediated by CXCR3 [55]. Whether CCL21 has an effect on neuronal activity is yet unknown, but this would be worthwhile to investigate. (For overview of all mentioned chemokines and receptors, their cellular sources and possible interactions see fig. 7-1).

In conclusion, CCL21 is expressed by stressed neurons, anterogradely transported and released at the synapse. Microglia expressing CXCR3 respond to CCL21 and might trigger neuroinflammatory processes. Since the process of microglia activation and neuroinflammation comprises neuroprotective as well as neurodetrimental aspects, the final effect of CCL21-release remains to be explored.

LPS-stimulated and ovalbumin processing microglia express CCR7 and respond to CCL21

Although expression of CCR7 was not found in ischemic brain and microglia under normal culture conditions, it was hypothesized that microglia might be able to express this receptor under certain circumstances and respond to CCL21. This hypothesis was based on the fact that microglia are thought to be potential APCs [2,3] and that professional APCs, like dendritic cells and macrophages, express CCR7 when they encounter, process and present antigens [22,49]. In other studies it was previously shown that bacterial cell wall components like LPS and OmpA induce CCR7 expression in DCs and macrophages [31,48,49,54].

In the **fifth** chapter, expression of CCR7 was studied in microglia that were exposed to LPS. PCR and Q-PCR experiments revealed a dramatic increase (≥100 times) of CCR7 mRNA expression after LPS treatment. In these experiments CCR7 was already detectable after 2 hours and remained present up to 24 hours after stimulation. In addition, these findings were verified by *in situ* hybridization experiments that showed increased expression of CCR7 in LPS-stimulated microglia. In order to provide a more direct link between antigen presentation and expression of CCR7, microglia were also exposed to ovalbumin. Following exposure to and uptake of ovalbumin an increase in microglial CCR7 mRNA expression was observed.

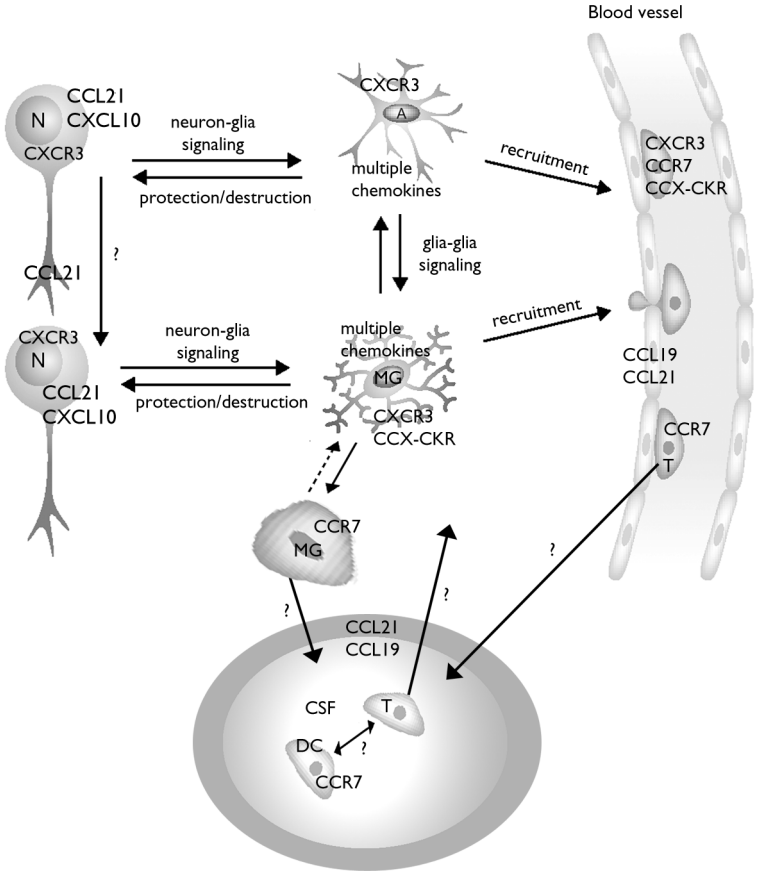

Figure 7-1 Intercellular communication in the CNS mediated by CCL21 and its receptors.

In this thesis CCL21 was found to be expressed, transported along the axon and released by endangered neurons. The receptor for CCL21, CXCR3, was found in astrocytes and microglia, suggesting possible neuron-glia communication. Since neurons also express CXCR3, CCL21 might as well have a direct effect on neuronal activity. Expression of another CXCR3 ligand, CXCL10, has been described in neurons [56], astrocytes and microglia [10,25,44,50-52] and might also be a mediator of neuron-glia signaling as well as glia-glia communication. Activation of glial cells by neuronal chemokines results in the production of factors that can either be neuroprototective or neurodetrimental. In addition, astrocytes and microglia express multiple chemokines that attract other glial cells and recruit leukocytes across the BBB.

Expression of the main receptor for CCL21, CCR7, was not found in CNS cells under normal culture conditions. CCR7 was found expressed in microglia under circumstances during which microglia developed into APC-like cells. Based on existing literature [7,21,34] it is suggested that antigen-loaded and CCR7^{+} microglia enter the CSF and possibly migrate to CCL19 and CCL21 in the cervical lymphnodes. Here they activate naïve or primed T-cells that subsequently enter the brain via the CSF and circulation. CCX-CKR, a CCL21 binding chemokine receptor-like protein, was found to inhibit CXCR3 mediated migration and might play a keyrole in regulating the trafficking/retention of leukocytes like T-cells and dendritic cells.

Regarding expression of CCR7 protein, this study was limited by the fact that no commercially available antibodies for murine CCR7 are present at the moment. This was circumvented by performing chemotaxis assays, which demonstrated CCR7-mediated migration of LPS stimulated microglia obtained from CXCR3 knockout mice. Based on these experiments the presence of CCR7 protein in LPS-stimulated microglia seems very likely. Hence, microglia are, under certain inflammatory conditions, able to express CCR7, which supports the assumption that microglia can become APCs.

CCR7 is expressed in the early stages of EAE

Expression of CCR7 was also examined in CNS tissue of mice suffering from EAE, an animal model for MS. This model displays a clear neuroinflammatory component and strong microglia activation. It was hypothesized that development of EAE might also involve upregulated CCR7 expression. Spinal cord and brain tissue obtained from mice displaying different clinical scores and CCR7 mRNA expression were analyzed by PCR and Q-PCR.

Interestingly, a marked increase in CCR7 mRNA expression was already found in tissue from animals with low clinical scores, thus at the onset of EAE pathology. Unfortunately, it was not possible to detect CCR7 in early-stage brain and spinal cord sections by *in situ* hybridization. During EAE, microglia activation was observed from the early stages on throughout the whole course of the disease. In contrast, infiltration of leukocytes was observed only at later stages of EAE[17]. As CCR7 expression has primarily been described in infiltrating cells, but not in CNS resident cell types, this might indicate that activated microglia are among the $CCR7^+$ cell population at the early stages of EAE. This is supported by a very recent study on MS brain material, which revealed upregulated CCR7 expression in MHC^+ myeloid cells, and microglia-like cells [34].

Functional meaning of CCR7 expression in microglia

As mentioned, expression of CCR7 in microglia was examined since activated microglia are known to resemble APCs, like DCs, which express CCR7 during maturation. Therefore, expression of CCR7 in microglia after exposure to LPS or ovalbumin is in support of the idea that microglia can be APCs. Consequently, CCR7 expression would enable microglia to migrate towards sources of its ligands, CCL19 and/or CCL21 in order to participate in ongoing inflammatory reactions.

Regarding CCR7 ligands in the CNS, few studies have reported expression of CCL21 and/or CCL19 in different brain pathologies. Besides our own data showing neuronal CCL21 expression after ischemia [14], CCL19 and CCL21 have been identified in neuroinflammatory conditions, like MS and its rodent model EAE.

Increased levels of both CCL19 and CCL21 have been found in the CSF and choroid plexus epithelial cells from MS patients [34,41]. In EAE, CCL19 and CCL21 was detected in venules of the BBB [4] and CCL19$^+$ cells have been found among the accumulating immune cells in EAE lesions [20].

The fact that microglia express two different receptors for CCL21 under varied circumstances, suggests that microglia can respond differentially to CCL21, dependent on the environmental stimuli. Thus, local and high levels of CCL21 that, for instance, can be provided by endangered neurons, trigger microglia activation and migration via activation of CXCR3 (low CCL21 affinity). On the other hand, secreted CCL19 and/or CCL21 in more "diffuse" CNS pathologies like MS and EAE could activate CCR7$^+$ (high CCL21 affinity) microglia in the direct surroundings but also at greater distance and induce migration towards the same sites as infiltrating immune cells. Since activated microglia are potential APCs they might -by retaining and presenting antigens- restimulate primed memory T-cells and initiate and/or maintain inflammation in the CNS.

Besides sources of CCL19/CCL21 in the CNS, these chemokines are also produced at high levels in the peripheral lymphnodes. In addition, there is good evidence that CSF drains to the cervical lymphnodes via cranial and spinal nerves [16]. Based on these two facts, it has been suggested that CCR7$^+$ microglia might leave the brain and migrate to the cervical lymphnodes. Here they would subsequently present antigens and prime naïve T-cells. In line with this idea, studies demonstrating that labeled antigens or DCs injected into the brain, either in the ventricles or parenchyma, can be traced in the cervical lymphnodes have been published [28,33,36]. Regarding the findings of increased CCR7 expression at the early stage of EAE, it is suggested that CCR7$^+$ APCs (including activated microglia) are involved in initiating the disease by activation of T-cells in the cervical lymphnodes. This relatively new and speculative idea, is supported by recent studies that reported:

1) Presence of myelin-containing CCR7$^+$ cells in cervical lymphnodes of EAE primates and MS patients [21]

2) Presence of CCR7$^+$ APCs (microglia or DCs) in MS brain parenchyma [34]

3) Presence of CCR7$^+$ DCs in CSF of MS patients [34]

4) Suppressed "cryolesion-induced aggravation" of EAE brain damage after removal of cervical lymphnodes [42]

In conclusion, it was demonstrated that microglia express CCR7 after LPS treatment, after exposure and processing of ovalbumin and at the early stage of EAE. This indicates differential microglial responses towards CCL21 and is in support of the idea that microglia can become APCs. Moreover, these results indicate that microglia can be targeted to sources of CCL19 and CCL21 in the CNS, CSF or

cervical LNs. Here microglia might play an important role in the activation of primed or naïve T cells and thus the initiation and maintenance of inflammatory processes in the CNS.

CCX-CKR inhibits CXCR3-mediated migration

The **sixth** chapter of this thesis describes a study that was performed in order to examine the effect of the chemokine receptor-like protein CCX-CKR on CXCR3 mediated chemotaxis. As demonstrated in this thesis, human microglia show functional activity of CXCR3 in response to CCL21. In contrast, recombinant systems expressing hCXCR3 are consistently found not to respond to hCCL21. We therefore suggest that the cellular background of human microglia provides, besides CXCR3, a factor that enables these cells to respond to CCL21. One of these factors might be the presence of additional chemokine receptors. For instance, it is known that chemokine receptors, like other GPCRs can form hetero- as well as homodimers, which can alter GPCR signaling properties.

CCX-CKR is a chemokine receptor-like protein that was reported to bind CCL19, CCL21 and CCL25 with high affinity [26]. Because expression of this receptor was also reported in the CNS [26] and particularly in microglia (own unpublished finding)[24], it was hypothesized that in human microglia CCX-CKR might alter CXCR3 signaling properties and render them responsive to CCL21. This hypothesis was tested by constructing recombinant HEK293 cell lines expressing CXCR3, CCX-CKR or both receptors and examination of migration in response to CCL21, CXCL9 and CXCL10.

Surprisingly, cells expressing both receptors did not respond to any of the ligands, whereas cells expressing CXCR3 showed typical migration towards CXCL9 and CXCL10. These results were exactly the opposite of what would be expected based on the hypothesis and, in addition, raised new intriguing questions such as: Why does the presence CCX-CKR abolish CXCR3 mediated migration and would this also be the case in primary cells? In order to answer these questions the effect of CCX-CKR expression was investigated at multiple levels.

First, the effect of CCX-CKR on changes in the actin cytoskeleton were examined. It was demonstrated that, in contrast to CXCR3 expressing cells, cells expressing CCX-CKR contained less F-actin and showed no increase in actin polymerization and filopodia formation after CXCL10 stimulation. Since actin polymerization is essential for chemotaxis this observation is line with the abolished CXCR3-mediated chemotaxis in CCX-CKR expressing cells. Second, the effect of CCX-CKR on CXCR3 ligand binding was examined. It was shown that the presence of CCX-CKR does not alter CXCR3 binding properties. Finally, since CCX-CKR inhibited CXCR3-mediated migration without the presence of CCX-CKR ligands, it was

hypothesized that CCX-CKR might exhibit constitutive activity. Two different parameters for constitutive activity were measured: accumulation of InsP and activation of NF-κB. However, both parameters were not affected by CCX-CKR expression and additional studies should be performed to further address this issue.

CCX-CKR expression in T-cells is related to their activation state

As mentioned, it would be interesting to explore the effects of CCX-CKR migration in primary cells. T-cells represent an important population of chemokine receptor expressing cells in the immune system and expression of CXCR3 in naïve $CD8^+$ and activated $CD4^+$ T-cells has been demonstrated [37,43]. Therefore, T-cell expression levels of CCX-CKR and CXCR3 were determined in both non-stimulated and PHA/IL-2 activated T-cells. A significant decrease in CCX-CKR mRNA expression was observed in activated human T-cells, whereas CXCR3 mRNA levels remained unaltered.

Unstimulated T-cells do not migrate towards CXCL10 despite CXCR3 expression

Migration of both unstimulated and PHA/IL-2 stimulated T-cells was examined in response to CXCL10. Whereas unstimulated T-cells (expressing both CXCR3 and CCX-CKR) did not migrate, activated T-cells (expressing CXCR3 and reduced CCX-CKR) showed typical chemotaxis, which thus resembled abolished migration in co-transfected HEK293 cells.

Functional meaning of CCX-CKR

Since CCX-CKR affects CXCR3-mediated migration in recombinant cells and since CCX-CKR expression in T-cells appears to depend on their activation state we suggest that this receptor might be important in regulating CXCR3-mediated migration of T-cells and retain these cells in the lymphnodes until activation. Furthermore, since a similar decrease in CCX-CKR mRNA expression for maturing dendritic cells was described [26], CCX-CKR, might also be involved in retaining immature dendritic cells in peripheral tissues. Accordingly, CCX-CKR might be a novel target for future therapeutic intervention in inflammatory disease and expression levels of CCX-CKR in other immune cells should be explored.

Although relevant to our knowledge on cell migration mechanisms and its related biological aspects (development, inflammatory reactions, tumor metastasis etc), few studies have addressed mechanisms involved in migration arrest. Among these mechanisms are chemoattractant receptor desensitization and receptor internalization [12,40,47]. Here, a novel mechanism was presented by which CXCR3- mediated migration is prevented by expression of the chemokine receptor-like protein CCX-

CKR. Despite the fact that the exact underlying signaling cascade is still unclear, changes in actin distribution are most likely to be involved. In respect to human microglia, the question why these cells show CXCR3 mediated activity in response to CCL21 remains to be answered, as CCX-CKR did not induce CXCR3 responsiveness to CCL21.

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